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Allosteric Modulation of the Calcium-sensing Receptor by γ-Glutamyl Peptides

INHIBITION OF PTH SECRETION, SUPPRESSION OF INTRACELLULAR cAMP LEVELS, AND A COMMON MECHANISM OF ACTION WITH L-AMINO ACIDS[*][S]

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γ-Glutamyl peptides were identified previously as novel positive allosteric modulators of $Ca^{2+}{}_o$-dependent intracellular Ca^{2+} mobilization in HEK-293 cells that bind in the calcium-sensing receptor VFT domain. In the current study, we investigated whether γ-glutamyl-tripeptides including γ-Glu-Cys-Gly (glutathione) and its analogs *S*-methylglutathione and *S*-propylglutathione, or dipeptides including γ-Glu-Ala and γ-Glu-Cys are positive allosteric modulators of $Ca^{2+}{}_o$-dependent $Ca^{2+}{}_i$ mobilization and PTH secretion from normal human parathyroid cells as well as $Ca^{2+}{}_o$-dependent suppression of intracellular cAMP levels in calcium-sensing receptor (CaR)-expressing HEK-293 cells. In addition, we compared the effects of the potent γ-glutamyl peptide *S*-methylglutathione, and the amino acid L-Phe on HEK-293 cells that stably expressed either the wild-type CaR or the double mutant T145A/S170T, which exhibits selectively impaired responses to L-amino acids. We find that γ-glutamyl peptides are potent positive allosteric modulators of the CaR that promote $Ca^{2+}{}_o$-dependent $Ca^{2+}{}_i$ mobilization, suppress intracellular cAMP levels and inhibit PTH secretion from normal human parathyroid cells. Furthermore, we find that the double mutant T145A/S170T exhibits markedly impaired $Ca^{2+}{}_i$ mobilization and cAMP suppression responses to *S*-methylglutathione as well as L-Phe indicating that γ-glutamyl peptides and L-amino acids activate the CaR via a common mechanism.

The extracellular calcium-sensing receptor (CaR)[3] is a multi-modal class C G-protein coupled receptor that is widely distributed in mammalian tissues (1). It controls the plasma Ca^{2+} ion concentration by mediating negative feedback regulation of parathyroid hormone secretion as well as $Ca^{2+}{}_o$-induced inhibition of Ca^{2+} reabsorption in the distal nephron (review: (2)). Analysis of the receptor's physiological significance has been based on its tissue distribution, analyses of its actions in cultured cells and tissues, and demonstrations of the impact of global knock-outs (3) and, more recently, conditional knock-outs in endocrine and mesenchymal tissues (4). These studies demonstrate that the CaR is a pluripotent sensor, regulator and modulator. In addition to regulating whole body calcium metabolism, it senses nutrient signals in the gastrointestinal tract (reviews: Refs. 5, 6), modulates synaptic transmission (7) and cellular differentiation in mesenchymal tissues (4), as well as engraftment of stem cells in specific niches (8), and organogenesis *e.g.* in the lung (9) and central nervous system (10, 11). Accompanying this remarkable plasticity, the CaR, like several other class C GPCRs, binds multiple physiologically relevant ligands to control intracellular signaling pathways (Reviews: Refs. 12, 13).

In addition to sensing multivalent cations including Ca^{2+} and Mg^{2+}, the CaR is modulated by various L-amino acids (review: Ref. 1). Based on chimeric receptor and mutational analyses, L-amino acids bind in the receptor N-terminal Venus Fly Trap (VFT) domain (14) and the effects of L-amino acids are selectively impaired by a double mutant (T145A/S170T), which exhibits normal $Ca^{2+}{}_o$-sensing (15). Comparative molecular modeling of the mGlu-1 and CaR VFT domain ligand binding surfaces indicates that, whereas the amino acid side-chain binding region is tightly defined by a cluster of positively charged residues in mGlu-1 and other mGlus (16, 17) it is relatively unrestricted in the CaR and closely related class C GPCRs including GPRC6A and T1R1 (18). The larger side-chain binding surfaces in this subgroup of receptors appear to explain, at least in part, their notable promiscuity for various sub-classes of amino acids (reviews: Refs. 19, 20) and has led to the successful prediction that small peptides that retain α-amino and α-carboxylate functional groups at their N termini, including the tri-peptide glutathione would bind and activate the CaR expressed in HEK-293 cells (21). Building on this observation, γ-glutamyl peptides were recently found to be potent taste enhancers (22) raising the possibility that they may be physiologically im-

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[3] The abbreviations used are: CaR, calcium-sensing receptor; GPCR, G protein-coupled receptor; SMG, *S*-methylglutathione.

portant receptor modulators in various tissues in which the CaR is expressed.

To further evaluate the physiological significance of these observations we have now tested the effects of the γ-glutamyl-tripeptides γ-Glu-Cys-Gly (glutathione), *S*-methylglutathione and *S*-propylglutathione, and dipeptides γ-Glu-Ala and γ-Glu-Cys, on extracellular $Ca^{2+}{}_o$-induced intracellular Ca^{2+} mobilization and suppression of cAMP levels in CaR-expressing HEK-293 cells. We then extended the investigation to consider the effects of these peptides on intracellular Ca^{2+} mobilization and PTH secretion in normal human parathyroid cells, and whether a double mutant T145A/S170T that selectively impairs amino acid-dependent activation of the CaR (15) might also impair the actions of γ-glutamyl peptides. The results indicate that various γ-glutamyl peptides including glutathione and its metabolite *S*-methylglutathione are potent positive allosteric modulators of the CaR that promote $Ca^{2+}{}_i$ mobilization and lower intracellular cAMP levels in CaR-expressing HEK-293 cells and promote $Ca^{2+}{}_i$ mobilization and suppress PTH secretion in normal human parathyroid cells. Results of a comparative analysis of HEK-293 cells that express either the wild-type or double mutant T145A/S170T CaR indicate that, distinct from $Ca^{2+}{}_o$ and phenylalkylamines, γ-glutamyl peptides and L-amino acids modulate the receptor via a common mechanism linking binding of L-amino acids or γ-glutamyl peptides at closely related sites in the VFT domain to a characteristic change in receptor conformation that couples to the modulation of cytoplasmic Ca^{2+} and cAMP levels.

EXPERIMENTAL PROCEDURES

Cell Culture of Control and Stably Transfected HEK-293 cells—Human embryonic kidney (HEK)-293 cells stably expressing the CaR (HEK-CaR cells), untransfected HEK-293 cells, and HEK-293 cells that stably expressed a double mutant form of the CaR (HEK-CaR-T145A/S170T cells) were thawed from frozen stocks and cultured in DMEM (Invitrogen)/10% fetal bovine serum as described previously (15). In general, cultured cells were studied between passage numbers 4 and 20. For microfluorimetry experiments, HEK-293 cells were transferred onto sterilized 15-mm diameter coverslips in 24-well plates and cultured for a further 24–48 h.

Preparation of Human Parathyroid Cells—Samples from normal human parathyroid auto-transplants were obtained during thyroid surgery at the Royal North Shore Hospital and North Shore Private Hospital, St Leonards, New South Wales and the Mater Misericordiae Hospital, North Sydney, New South Wales, Australia. All procedures were performed under guidelines established by the relevant human research ethics committees, and all patients provided written informed consent for the use of the tissue for experimental purposes.

Parathyroid tissue was transported to the laboratory in MEM that contained 1.25 mM $CaCl_2$. Upon arrival in the laboratory, it was either used immediately or, more typically, stored overnight in MEM at 4 °C as described previously (23). Prior to collagenase digestion, parathyroid tissue retains viability under these conditions (24). For digestion, it was transferred to MEM that contained 1 mg/ml collagenase and 0.1 mg/ml DNase I. After brief oxygenation it was incubated at 37 °C for 20 min. The enzyme suspension was then decanted and the parathyroid tissue was transferred into 5 ml of MEM that contained 1 mg/ml of bovine serum albumin (BSA). The tissue was then subjected to trituration (10–15 times) through the tip of a disposable 5-ml syringe (no needle attached). The cloudy suspension containing clumped parathyroid cells was then passed through a 200-μm pore size nylon filter, sedimented (70 × *g*, 2.5 min), and the cell pellet gently resuspended then washed twice with 5 ml of BSA-containing MEM. It was finally resuspended in 2 ml of bovine serum albumin-containing MEM. Remaining pieces of undigested parathyroid tissue were then incubated for a further 20 min at 37 °C in fresh medium containing collagenase and DNase I. The enzyme suspension was once more decanted, and the parathyroid tissue subjected to trituration and centrifugal isolation as above.

Amino acid and Peptide-containing Solutions—Stock amino acid-containing solutions were routinely made up in physiological saline at 50 or 100 mM and stored at −20 °C then diluted in amino acid-free physiological saline as required. Di-peptide and tri-peptide containing solutions, on the other hand, were made up fresh in physiological saline and used on the day of experiment. The control physiological saline solution (PSS) used in all microfluorimetry experiments had the following composition: 125 mM NaCl, 4.0 mM KCl, 0.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 20 mM HEPES (NaOH), 0.1% D-glucose (pH 7.4).

Microfluorimetry of HEK-293 Cells and Human Parathyroid Cells for Determination of Intracellular Ca^{2+} Concentration—HEK-293 cells that had been cultured on coverslips in 24-well plates were loaded with 5 μM fura2-AM in PSS that contained 1 mg/ml bovine serum albumin at 37 °C for 2 h in the dark. This solution was then removed and replaced by fresh albumin-containing PSS and the cells were incubated for a further 30 min to promote the conversion of fura-2 to its fully ionized, Ca^{2+}-binding form.

Suspensions of normal human parathyroid cells, which had been prepared by collagenase digestion, were loaded with 1 μM fura-2 AM for 20 min at 37 °C in MEM containing bovine serum albumin (1 mg/ml). The cells were sedimented (70 × *g*, 2.5 min) and then resuspended in albumin-free physiological saline solution.

Fura-2-loaded HEK-293 cells that had been cultured on coverslips or fura-2 loaded parathyroid cells, which readily attach to glass surfaces, were transferred into a superfusion chamber, placed in the light path of a Zeiss Axiovert fluorescence microscope (63× objective) and perifused with inorganic phosphate-free physiological saline solutions that were modified to contain various concentrations of $Ca^{2+}{}_o$, L-Phe, and γ-glutamyl di- and tri-peptides. A Lambda DG-4 150 watt Xenon light source (Sutter) was programmed to alternate between two excitation wavelengths (340 and 380 nm) at a frequency of 1 s^{-1}. Areas of interest were selected and digital images were then captured and downloaded using a high resolution AxioCam camera controlled by Stallion SB.4.1.0 PC software (Zeiss, Australia). Fura-2-loaded cells were imaged at 510 nm. For concentration-response analysis, fura-2 ratio

data were integrated and then either plotted directly or expressed as a ratio of a control response (*e.g.* L-Phe or R-467).

Detection of Changes in Intracellular cAMP Levels in CaR-expressing HEK-293 Cells—HEK-CaR, HEK-CaR-T145A/S170T, or control HEK-293 cells were seeded onto 15-mm coverslips in 24-well plates for 24 h and then transfected for 48 h with pcDNA3.1 containing the cAMP bio-sensor CFPnd-EPac1-cpVenus (EPac1; the generous gift of Dr Kees Jalink, Netherlands Cancer Institute) using Lipofectamine-2000 according to the manufacturer's instructions (Invitrogen). The media were replaced with PSS containing 0.5 mM Ca^{2+} for 30 min at 37 °C, and coverslips were then transferred to a chamber placed in the light path of a Zeiss Axiovert epi-fluorescence microscope (63× objective) and perifused with PSS that contained various concentrations of $Ca^{2+}{}_o$, L-Phe, γ-glutamyl di- or tri-peptides, or the type-II calcimimetic NPS R-467 as required. Epac1-transfected cells were excited continuously with light centered on a wavelength of 436 nm using the Lambda DG-4 light source and emitted light corresponding to CFP and Venus YFP emissions were sampled at 1s intervals using filters centered on 488 nm (CFP) and 528 nm (Venus-YFP) to permit the detection of cAMP-dependent changes in FRET. Cells of interest were selected and digital images were captured and downloaded as described above. The ratios of the fluorescence readings at 488 nm and 528 nm were plotted as a function of time after correcting for background in the absence of cells.

Determination of PTH Secretion from Perifused Human Parathyroid Cells—Perifusion of normal human parathyroid cells was undertaken in low molecular mass (4–5 kDa) cut-off gel filtration micro beads so that intact PTH (~9 kDa) would appear in the void volume as described previously (23, 25). Gel filtration media were pre-equilibrated with physiological saline: 125 mM NaCl, 4.0 mM KCl, 1.25 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.8 mM Na_2HPO_4, 20 mM HEPES (NaOH), 0.1% D-glucose (pH 7.4) that contained 1× basal amino acid mixture (total concentration 2.8 mM; (26)), and 1 mg/ml bovine serum albumin. Around 40,000–50,000 cells were loaded onto the surface of a 0.4-ml bed volume of Bio-gel P-4 (nominal exclusion limit 4 kDa) and then gently covered with a 0.4-ml bead volume of Sephadex G-25 (nominal exclusion limit 5 kDa) in a small perifusion column. Tubing connections were then established downstream to a peristaltic pump and up-stream to a reservoir and the column was suspended in a water bath (37 °C) and perifused at 1.5 ml/min with 37 °C equilibrated control physiological saline that contained the 1-fold L-amino acid mixture and 1 mg/ml bovine serum albumin. Routinely, 2-min (*i.e.* 3 ml) samples were collected into tubes immersed in an ice bath and then transferred to dry ice. As required, solutions were changed to permit variations in the concentrations of $Ca^{2+}{}_o$, amino acids or γ-glutamyl peptides. All samples were stored at −80 °C until analysis of intact human PTH using an Immulite 2000 autoanalyzer. For estimates of steady-state levels of PTH secretion, the first 2-min samples of all treatment periods, during which the cells were re-equilibrating, were routinely ignored and the means of the second and third samples were used for plotting the concentration-response relationships and curve-fit analysis.

Statistical Analysis and Curve Fitting—Microfluorimetry experiments yielded single cell data-sets consisting of fluorescence excitation ratios (F_{340}/F_{380}) and the corresponding times (s) since the start of the experiment. The fluorescence ratio data were integrated and the baseline responses corresponding to an initial control period were subtracted to yield activator- and concentration-dependent receptor-response values. The resulting data were plotted unmodified or expressed as ratios of control responses. For γ-glutamyl peptide responses, either 10 mM L-Phe or 5 μM NPS R-467 were used as controls. Experimental data were plotted using DeltaGraph 5.0 or GraphPad Prism 5.0b and curve-fitting was performed using the Hill equation: $r = b + (a\text{-}b)\ C^n/(e^n+C^n)$ where r = response, a = maximum response, b = basal response, C = extracellular Ca^{2+} concentration (in mM), e = EC_{50} (the $Ca^{2+}{}_o$ concentration that induced a half-maximal response) and n = Hill coefficient. PTH secretion data were fitted to the following equation: $S = a - (a-b)\ C^n/(i^n+C^n)$ where S = secretory response, a = maximum secretory response, b = basal secretory response, C = extracellular Ca^{2+} concentration (in mM), i = IC_{50} (the $Ca^{2+}{}_o$ concentration that yielded half-maximal inhibition) and n = Hill coefficient. The data were expressed routinely as means ± S.E. (number of experiments). Statistical comparisons between treatments within PTH secretion data sets were performed using Student's paired t test (two-tailed) in SPSS Statistics 17.0. Statistical comparisons between the effects of positive allosteric modulators on pEC_{50} values, maximum responses, or specific data points in $Ca^{2+}{}_o$ concentration-response curves were performed using Student's unpaired t test (two-tailed) in *SPSS*. Statistically significant comparisons were accepted at $p < 0.05$.

RESULTS

Effects of γ-Glutamyl Peptides on $Ca^{2+}{}_o$-dependent Intracellular Ca^{2+} Mobilization in CaR-expressing HEK-293 Cells—No effects of γ-glutamyl peptides were observed in untransfected HEK-293 cells (not shown). At extracellular Ca^{2+} ($Ca^{2+}{}_o$) concentrations below 1.0 mM, the amino acid L-Phe (10 mM) and the tripeptide glutathione and two analogs *S*-methylglutathione and γ-Glu-Ala had no effect on intracellular Ca^{2+} ($Ca^{2+}{}_i$) mobilization in HEK-CaR cells. For example, in the presence of 0.5 mM $Ca^{2+}{}_o$, none of the peptides was effective at concentrations up to 100 μM (not shown). At $Ca^{2+}{}_o$ concentrations above 1.0 mM, however, L-Phe and glutathione acutely and reversibly stimulated oscillations in the intracellular Ca^{2+} concentration in HEK-CaR cells and a $Ca^{2+}{}_o$ concentration of 2.5 mM was selected for experiments comparing the efficacies and potencies of γ-glutamyl peptides including the dipeptides γ-Glu-Ala and γ-Glu-Cys, and tripeptides, glutathione and *S*-methylglutathione (Fig. 1*A* and Table 1).

All four γ-glutamyl peptides were effective positive allosteric modulators of $Ca^{2+}{}_i$ mobilization in the presence of 2.5 mM Ca^{2+}. The order of potency for $Ca^{2+}{}_i$ mobilization was as follows (at $Ca^{2+}{}_o$ = 2.5 mM; Fig. 1, *B* and *C* and Table 1): *S*-methylglutathione > glutathione > γ-Glu-Ala > L-Phe. In separate experiments, similar potencies were observed for γ-Glu-Cys and γ-Glu-Ala (Table 1). Co-administration of the

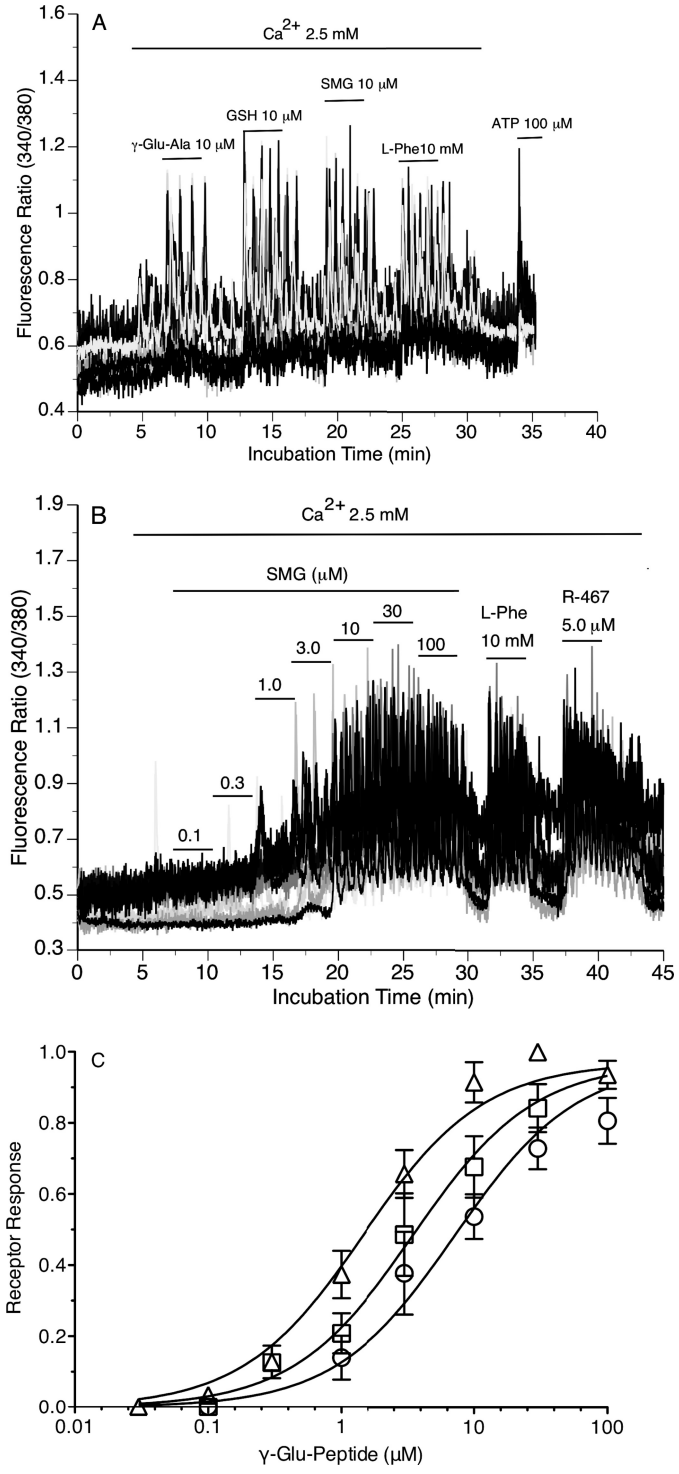

FIGURE 1. **Impacts of γ-glutamyl peptides on intracellular Ca^{2+} mobilization in CaR-expressing HEK-293 cells.** HEK-293 cells that stably express the CaR were loaded with fura-2AM and transferred into the light-path of a microfluorescence apparatus for analysis of intracellular Ca^{2+}. *A*, effects of various γ-glutamyl peptides including γ-Glu-Ala, glutathione (GSH) and SMG (all 10 μM) as well as the amino acid L-Phe in the presence of 2.5 mM Ca^{2+}; extracellular ATP was included as a positive control. The data shown were obtained from 10 cells in one experiment, representative of four independent experiments. *B*, effects of stepwise increases in the concentration of SMG (0.1–100 μM) in the presence of 2.5 mM Ca^{2+}; L-Phe and R-467 were included as controls. The data were obtained from 9 cells in one representative experiment ($n = 4$). *C*, concentration-response curves derived from experiments similar to, and including that, presented in *B*. Receptor response was obtained by integrating fluorescence ratio data at different activator concentrations in individual experiments and correcting for the baseline response ($n = 4$ for SMG; $n = 5$ for GSH; $n = 3$ for γ-Glu-Ala). The symbols are as follows: *circles*, γ-Glu-Ala; *triangles*, *S*-methylglutathione; *squares*, glutathione. The data were corrected for baseline.

TABLE 1

Potencies of γ-glutamyl peptides for Ca^{2+}_i mobilization in CaR-expressing HEK-293 cells

HEK-293 cells that stably express the CaR were loaded with fura-2AM and assayed for receptor-dependent intracellular Ca^{2+} mobilization by microfluorimetry. The data were obtained from cells perifused with physiological saline solution in the presence of 2.5 mM Ca^{2+}_o. In accompanying experiments, the EC_{50} for L-Phe was 1.1 ± 0.5 mM ($n = 4$).

γ-Glutamyl peptide	EC_{50} for peptide
	μM
S-Methylglutathione	1.7 ± 0.5 ($n = 4$)
Glutathione	3.9 ± 0.7 ($n = 4$)
γ-Glu-Cys	4.7 ± 0.9 ($n = 3$)
γ-Glu-Ala	4.8 ± 0.7 ($n = 3$)

recognized CaR-active amino acid, L-Phe and high potency γ-glutamyl peptide *S*-methylglutathione, at maximal concentrations *i.e.* 10 mM and 10 μM respectively, induced no additional effect when compared with either L-Phe or *S*-methylglutathione alone (not shown). On the other hand, at a Ca^{2+}_o concentration of 2.5 mM, maximally effective concentrations of *S*-methylglutathione and NPS R-467 exhibited significant positive interactions when compared with the effects of either compound alone (Fig. 2).

Effects of γ-Glutamyl Peptides on Ca^{2+}_o-dependent Intracellular Ca^{2+} Mobilization in Normal Human Parathyroid Cells—Of the amino acids and peptides that had been identified as positive allosteric modulators of Ca^{2+}_i mobilization in HEK-CaR cells, L-Phe, γ-Glu-Ala, glutathione, and *S*-methylglutathione, together with another glutathione analog, *S*-propylglutathione were selected for further studies of intracellular Ca^{2+} mobilization in fura-2 loaded normal human parathyroid cells. At Ca^{2+}_o concentrations at or below 0.5 mM, none of the compounds was effective. At Ca^{2+}_o concentrations greater than 1.0 mM, however, all of the compounds acutely mobilized intracellular Ca^{2+} (Fig. 3*A*). At a Ca^{2+} concentration of 1.5 mM, the concentration-response relations indicated that the γ-glutamyl peptides were effective in the micromolar concentration range with *S*-methylglutathione exhibiting a near maximal effect at around 2–5 μM (Fig. 3, *B* and *C*). The order of potency for intracellular Ca^{2+} mobilization was as follows: *S*-methylglutathione > glutathione > γ-Glu-Ala > *S*-propylglutathione (Fig. 3*C* and Table 2).

Effects of γ-Glutamyl Peptides on PTH Secretion from Normal Human Parathyroid Cells—We next tested the effects of γ-glutamyl peptides including γ-Glu-Ala, glutathione and *S*-methylglutathione (Fig. 4) on PTH secretion from perifused normal human parathyroid cells. All three peptides induced acute and reversible suppression of PTH secretion at a Ca^{2+}_o concentration of 1.0 mM (Fig. 4*A*) and exhibited concentration-dependent inhibition of secretion (Fig. 4*B*; $p < 0.01$ for the effect of 20 μM *S*-methylglutathione with respect to the pre- and post controls). For *S*-methylglutathione, the IC_{50} value was 4.8 ± 0.3 μM ($n = 3$; Fig. 4, *B* and *C*). For γ-Glu-Ala, the IC_{50} value was ~30 μM (supplemental Fig. S1). The results indicate that γ-glutamyl peptides and *S*-methylgluta-

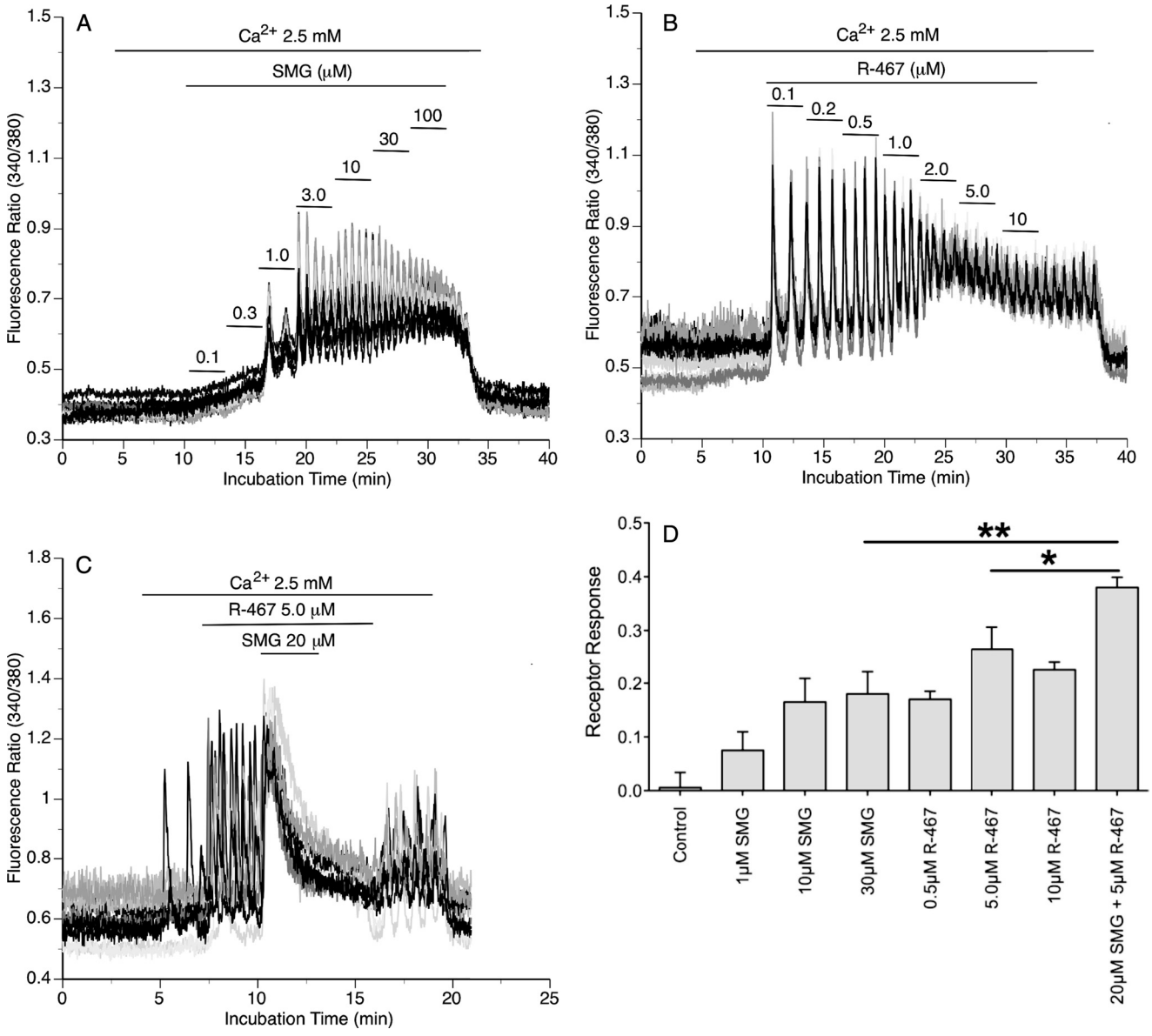

FIGURE 2. **Positive interaction between *S*-methylglutathione and phenylalkylamine NPS R-467.** HEK-293 cells that stably express the CaR (HEK-CaR cells) were loaded with fura-2AM and transferred into the light-path of a microfluorescence apparatus for analysis of intracellular Ca^{2+}. *A*, concentration-dependent activation of intracellular Ca^{2+} mobilization by the γ-glutamyl peptide SMG in 10 cells from a representative experiment. *B*, concentration-dependent activation of intracellular Ca^{2+} mobilization by NPS R-467 in 10 cells from a representative experiment. *C*, impact of a maximally effective concentration of SMG (20 μM) on $Ca^{2+}{}_i$ mobilization in cells exposed to a maximally effective concentration of R-467 (5 μM). A similar effect was observed when the order of application of the two positive allosteric modulators was reversed (not shown). *D*, positive interaction between maximally effective concentrations of SMG and R-467. *, $p < 0.05$; **, $p < 0.01$. The integrated response data in D were obtained in a minimum of four independent experiments in each case.

thione, in particular, are potent suppressors of PTH secretion raising the possibility that glutathione or an extracellular metabolite may act physiologically to modulate PTH secretion. In addition, *S*-methylglutathione or a structural analog may be of value in the treatment of various forms of hyperparathyroidism.

Impact of the Double Mutant T145A/S170T on $Ca^{2+}{}_i$ Mobilization Responses in CaR-expressing HEK-293 Cells to L-Phe and S-Methylglutathione—Chimeric receptor analysis previously localized the CaR L-amino acid (14) and glutathione (21) binding sites to the VFT domain and we previously identified T145A/S170T as a double mutant form of the VFT domain with near-normal $Ca^{2+}{}_o$-sensitivity but markedly impaired L-amino acid sensing (15). In the current study, we first confirmed that this mutant exhibited normal or near-normal $Ca^{2+}{}_o$ sensitivity in the absence of L-Phe or *S*-methylglutathione (Fig. 5, *A* and *B*). In the presence of 10 mM L-Phe, HEK-293 cells expressing the wild-type CaR exhibited markedly enhanced $Ca^{2+}{}_o$ sensitivity (Fig. 5*C*) whereas HEK-293 cells expressing the T145A/S170T double mutant CaR exhibited no change in $Ca^{2+}{}_o$ sensitivity (Fig. 5*D*) as reported previously (15).

We next tested the impacts of the potent γ-glutamyl peptide, *S*-methylglutathione on the $Ca^{2+}{}_o$ sensitivity of $Ca^{2+}{}_i$ mobilization in wild-type CaR and T145A/S170T CaR-expressing cells. In HEK-CaR cells, 10 μM *S*-methylglutathione induced a marked increase in $Ca^{2+}{}_o$ potency (Fig. 5*E*) but had little or no effect on $Ca^{2+}{}_o$ potency in HEK-293 cells express-

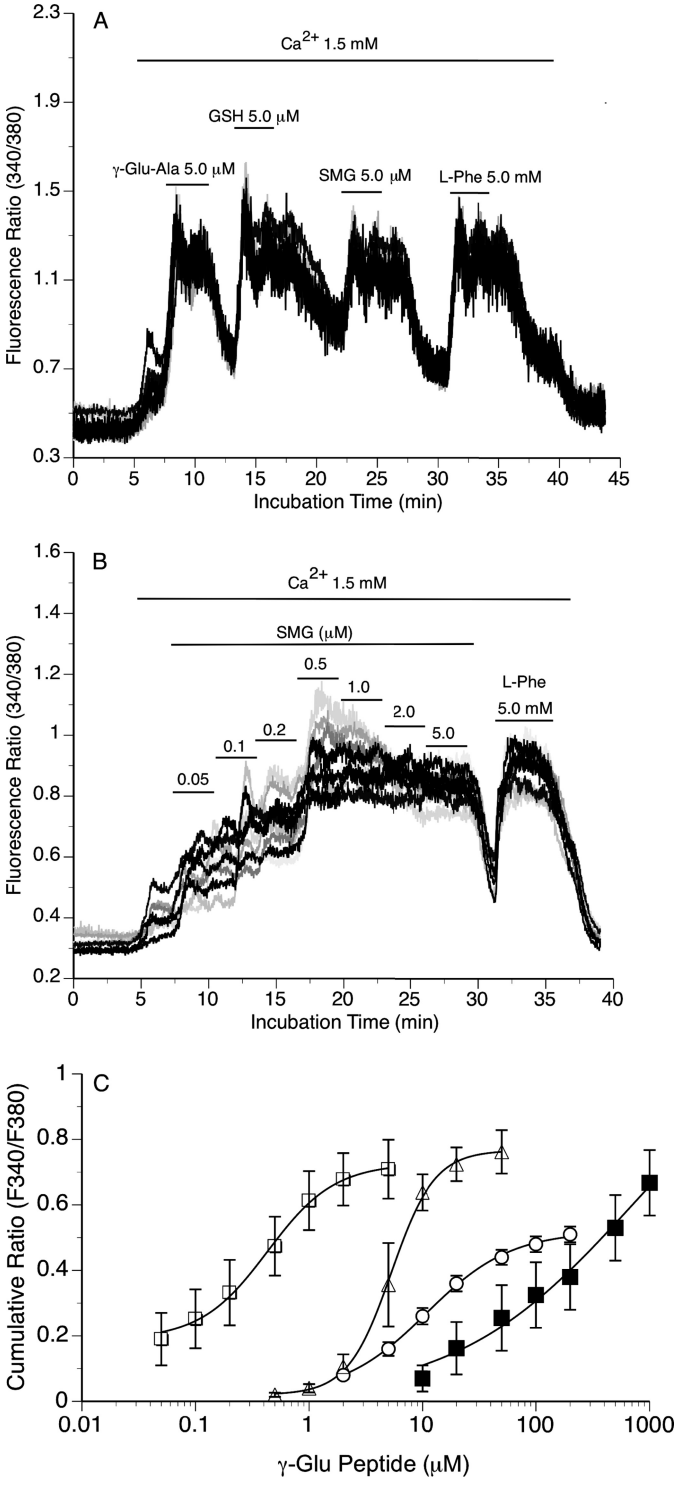

FIGURE 3. **Impacts of various γ-glutamyl peptides on intracellular Ca^{2+} mobilization in normal human parathyroid cells.** Normal human parathyroid cells were loaded with fura-2AM and transferred into the light-path of a microfluorescence apparatus for analysis of intracellular Ca^{2+}. *A*, effects of various γ-glutamyl-peptides (all 5 μM) as well as 5 mM L-Phe in the presence of a submaximal $Ca^{2+}{}_o$ concentration, 1.5 mM. Multiple single cell time-courses ($n = 10$) are shown from a representative experiment. *B*, effect of stepwise increases in SMG concentration on $Ca^{2+}{}_i$ in 9 single cells from a representative experiment; L-Phe was included as a control. *C*, concentration-response curves for various γ-glutamyl peptides derived from experiments similar to that shown in *B*. The numbers of independent experiments were as follows: glutathione ($n = 5$); γ-Glu-Ala ($n = 6$); SMG ($n = 5$); and *S*-propyl-glutathione (SPG; $n = 4$). The symbols are as follows: *open circles*, γ-Glu-Ala; *open triangles*, glutathione; *open squares*, SMG; *filled squares*, SPG.

TABLE 2

Potencies of γ-glutamyl peptides for $Ca^{2+}{}_i$ mobilization in normal human parathyroid cells

Normal human parathyroid cells were loaded with fura-2AM and assayed for receptor-dependent intracellular Ca^{2+} mobilization by microfluorimetry. The data were obtained from cells perifused with physiological saline solution in the presence of 1.5 mM $Ca^{2+}{}_o$.

γ-Glutamyl peptide	EC_{50} for peptide
	μM
S-Methylglutathione	0.38 ± 0.09 ($n = 5$)
Glutathione	5.4 ± 1.0 ($n = 5$)
γ-Glu-Ala	13.9 ± 5.7 ($n = 6$)
S-Propylglutathione	221 ± 107 ($n = 4$)

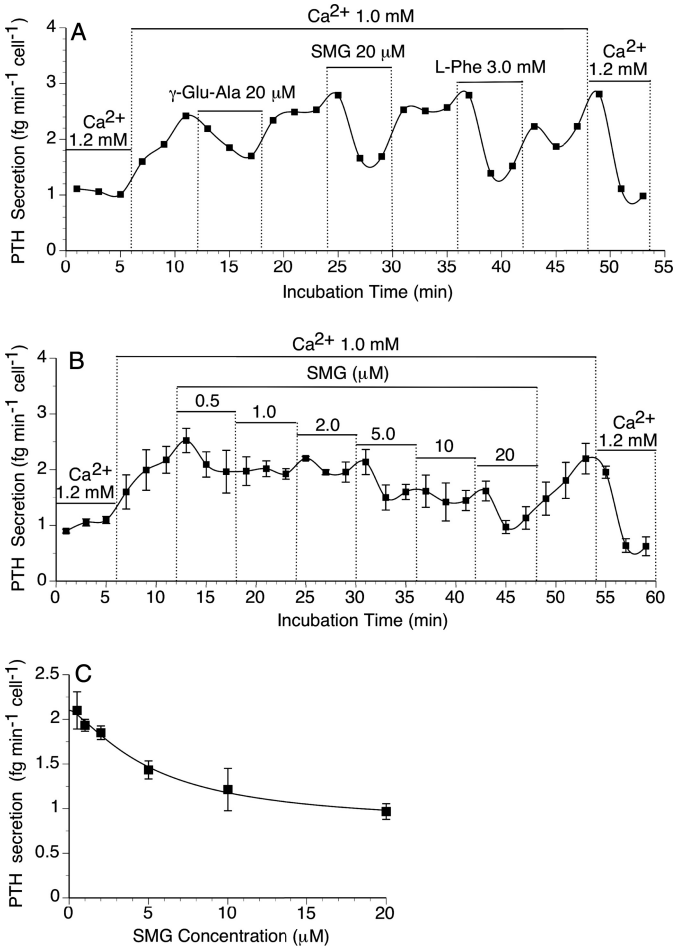

FIGURE 4. **Effects of γ-glutamyl peptides on PTH secretion from normal human parathyroid cells.** Normal human parathyroid cells were loaded into a perifusion column for the analysis of acute changes in PTH secretion as described under "Experimental Procedures." *A*, representative time course showing the acute effects of γ-Glu-Ala and SMG (both 20 μM) as well as L-Phe (3 mM) on PTH secretion; similar results were obtained in two other experiments. *B*, effect of stepwise increases in SMG concentration (0.5–20 μM) on PTH secretion. The data are means ± S.E. from three experiments. Student's *t* test analysis of comparisons between pre- and post-controls and 20 μM SMG were significant in both cases ($p < 0.01$; $p < 0.02$). *C*, concentration-response curve demonstrating the inhibitory effect of SMG on PTH secretion in the presence of 1.0 mM $Ca^{2+}{}_o$; the data were derived from the time-course experiments shown in *B* above ($n = 3$).

ing the CaR double mutant T145A/S170T (Fig. 5*F*). Consistent with these findings, concentration-response relations for the wild-type CaR and T145A/S170T double mutant demonstrated very similar $Ca^{2+}{}_o$ sensitivities in the absence of allosteric modulators (Fig. 6, *A* and *B*) and the EC_{50} values for

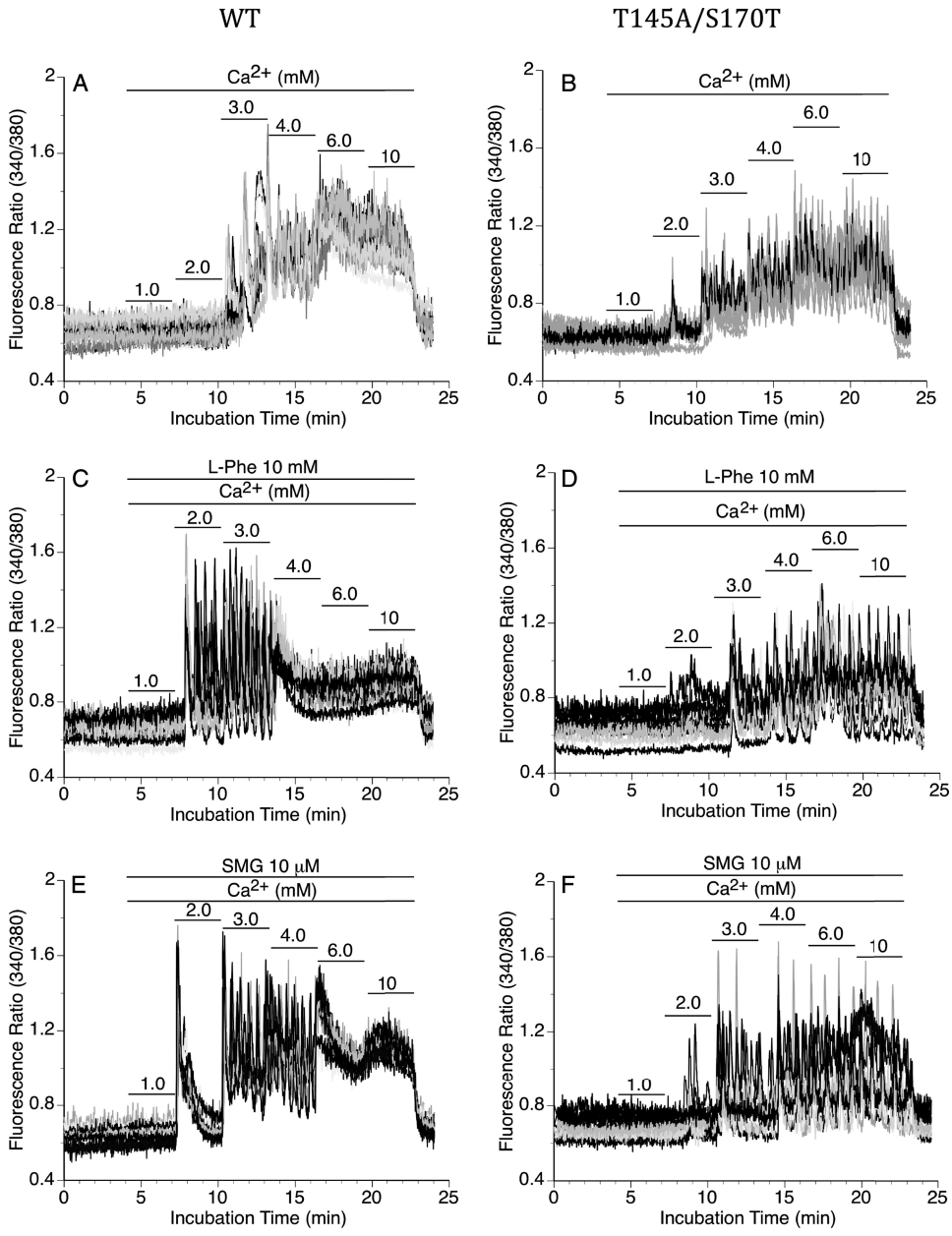

FIGURE 5. **Sensitivity of double mutant CaR (T145A/S170T) to modulation of $Ca^{2+}{}_o$-dependent $Ca^{2+}{}_i$ mobilization in HEK-293 cells by L-Phe or *S*-methylglutathione.** HEK-293 cells that stably expressed either the wild-type CaR (*left panels*: *A*, *C*, *E*) or the double mutant T145A/S170T (*right panels*: *B*, *D*, *F*) were loaded with fura-2AM as described under "Experimental Procedures" and then exposed to stepwise changes in $Ca^{2+}{}_o$ concentration in the absence of modulators (*panels A* and *B*) or the presence of 10 mM L-Phe (*panels C* and *D*) or 10 μM SMG (*panels E* and *F*). The data shown were obtained in single representative experiments (8–10 cells per experiment; 4–6 experiments per panel).

$Ca^{2+}{}_o$ were as follows: 3.9 ± 0.2 mM ($n = 4$) for HEK-293 cells that stably expressed the wild-type CaR and 3.6 ± 0.3 mM ($n = 4$) for HEK-293 cells that stably expressed the T145A/S170T double mutant CaR, respectively. Although the wild-type CaR exhibited markedly enhanced $Ca^{2+}{}_o$ sensitivity in the presence of 10 mM L-Phe (EC_{50} for $Ca^{2+}{}_o$ = 1.7 ± 0.3 mM; $n = 4$) or 10 μM *S*-methylglutathione (EC_{50} for $Ca^{2+}{}_o$ = 2.4 ± 0.3 mM; $n = 4$), the double mutant exhibited markedly impaired responses to both modulators (see Fig. 6, *A* and *B*). Thus, the EC_{50} values for $Ca^{2+}{}_o$ (in mM) were, respectively, 3.6 ± 0.3 ($n = 5$), 3.4 ± 0.2 ($n = 7$), and 3.5 ± 0.2 ($n = 7$) in the absence of modulators or in the presence of 10 mM L-Phe or 10 μM *S*-methylglutathione ($p = 0.78$ for L-Phe and $p = 0.82$ for *S*-methylglutathione with respect to control).

To further investigate the impacts of L-Phe and *S*-methylglutathione on the CaR double mutant T145A/S170T, we examined the effects of stepwise increases in the concentrations of L-Phe and *S*-methylglutathione in the presence of a submaximal $Ca^{2+}{}_o$ concentration, 2.5 mM using 5 μM NPS R-467 as a positive control (Fig. 7). In the case of the wild-type CaR, both L-Phe and *S*-methylglutathione induced robust concentration-dependent increases in $Ca^{2+}{}_i$ mobilization in the presence of 2.5 mM $Ca^{2+}{}_o$ (Fig. 7, *A* and *C*). In the case of the CaR double mutant T145A/S170T, however, the effects of

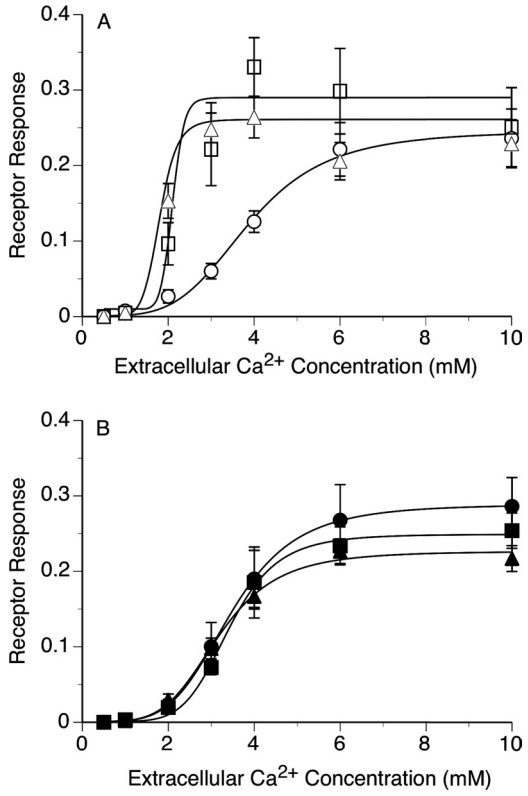

FIGURE 6. **Behavior of double mutant (T145A/S170T) CaR in $Ca^{2+}{}_o$ concentration-response analyses in the absence or presence of L-Phe or *S*-methylglutathione.** $Ca^{2+}{}_o$ concentration-response relations were derived from data obtained in experiments similar to, and including, those shown in Fig. 5. In *A*, the effects of 10 mM L-Phe (*open triangles*; $n = 4$) and 10 μM SMG (*open squares*; $n = 4$) are compared with control (*open circles*; $n = 4$) for HEK-293 cells that stably expressed the wild-type CaR. The differences between L-Phe or SMG and control were statistically significant at the following submaximal $Ca^{2+}{}_o$ concentrations: 2.0 mM ($p < 0.05$; $p = 0.05$); 3.0 mM ($p < 0.01$; $p < 0.01$); and 4.0 mM ($p < 0.01$; $p < 0.01$). In *B*, the effects of 10 mM L-Phe (*filled triangles*; $n = 4$) and 10 μM *S*-methylglutathione (*filled squares*; $n = 4$) are compared with control (*filled circles*; $n = 4$) for HEK-293 cells that stably expressed the CaR double mutant T145A/S170T.

both L-Phe and *S*-methylglutathione were attenuated (Fig. 7, *B* and *D*). Concentration-response relations for L-Phe and *S*-methylglutathione derived from experiments including those shown in Fig. 7 demonstrated marked impairment of $Ca^{2+}{}_i$ mobilization responses in HEK-293 cells expressing the T145A/S170T double mutant when compared with wild-type (Fig. 8) and similar results were obtained whether the data were expressed as integrated fluorescence ratio responses (Fig. 8) or, alternatively, as fractional responses with respect to a control, 5 μM R-467 (not shown).

Interestingly, the double mutant exhibited markedly suppressed maximum responses to L-Phe and *S*-methylglutathione but appeared to have little or no effect on the potencies of the modulators (Fig. 7, *B* and *D*; Fig. 8). These results suggest that the double mutant has little or no effect on modulator binding but instead impairs the coupling between $Ca^{2+}{}_o$ and positive allosteric modulators such as L-Phe and glutathione analogs that bind in the VFT domain.

Impacts of L-Phe and S-Methylglutathione on $Ca^{2+}{}_o$-dependent cAMP Suppression in HEK-293 Cells that Stably Express the Wild-type CaR or T145A/S170T Double Mutant—Finally, we investigated the impact of L-Phe, *S*-methylglutathione and elevated $Ca^{2+}{}_o$ on intracellular cAMP levels in HEK-CaR cells that were transfected with the cAMP reporter CFPnd-EPac1-cpVenus since the CaR is known to suppress cAMP levels in a G_i and $Ca^{2+}{}_i$-dependent manner (27). cAMP levels were first elevated by exposure to 5 μM forskolin and the cells were then exposed to one or more CaR activators in the continuing presence of forskolin. Elevated $Ca^{2+}{}_o$ induced a concentration-dependent inhibition of cAMP levels that appeared to reach a maximum at around 6 mM (Fig. 9*A*). Restoring $Ca^{2+}{}_o$ to the baseline level (0.5 mM) led to a prompt recovery in intracellular cAMP levels, which took the form of an overshoot following exposure to high $Ca^{2+}{}_o$ concentration (≥ 5 mM) (Fig. 9*A*), consistent with the phenomenon of sensitization or superactivation of adenylyl cyclase (review: Ref. 28). No effects of elevated $Ca^{2+}{}_o$, L-Phe, or *S*-methylglutathione were observed in control HEK-293 cells.

Consistent with their behaviors as positive allosteric modulators, L-Phe and *S*-methylglutathione were without effect on intracellular cAMP levels in HEK-CaR cells at a $Ca^{2+}{}_o$ concentration of 0.5 mM (not shown). In the presence of submaximal $Ca^{2+}{}_o$ concentrations (1.5 or 2.5 mM), however, 10 mM L-Phe and 10 μM *S*-methylglutathione acutely and reversibly suppressed cAMP levels (Fig. 9*B*), and the phenylalkylamine type-II calcimimetic NPS R-467 (0.2–5 μM) had a similar effect (not shown). We next tested the effects of L-Phe, S-methylglutathione, and elevated $Ca^{2+}{}_o$ on intracellular cAMP levels in HEK-293 cells that stably expressed the T145A/S170T CaR double mutant. The effects of 10 mM L-Phe and 10 μM *S*-methylglutathione were markedly attenuated, however, the effects of 5 mM $Ca^{2+}{}_o$ (Fig. 9*C*) and 5 μM R-467 (not shown) were unaffected.

DISCUSSION

The CaR has several binding sites for Ca^{2+} ions and other multivalent cations in its VFT (29) and heptahelical (14, 30) domains. These so-called type-I (cationic) calcimimetics are generally regarded as full agonists of the receptor since they activate multiple downstream signaling pathways linked to the activation of $G_{q/11}$, $G_{i/o}$ or $G_{12/13}$ (reviews: Refs. 12, 13, 31). Thus, their binding site(s) define the location(s) of the receptor orthosteric site(s). In addition, the CaR binds so-called type-II calcimimetics, which are typically uncharged organic compounds that act as positive allosteric modulators. Two main sites for the binding of positive allosteric modulators have been identified: one in the heptahelical domain for type-II calcimimetics such as the phenylalkylamines NPS R-467 and cinacalcet (30, 32); and one or more in the VFT domain for L-amino acids such as L-Phe or L-Trp (14) and glutathione and analogs (21). Previous work suggested that the L-amino acid binding site is selectively disabled by the double mutant T145A/S170T (15).

In the current study, we provide further evidence of the efficacy of γ-glutamyl peptides as positive allosteric modulators of $Ca^{2+}{}_o$-induced $Ca^{2+}{}_i$ mobilization in CaR-expressing HEK-293 cells (Fig. 1 and Table 1) and demonstrate their efficacy in normal human parathyroid cells (Fig. 3 and Table 2). In addition, we found that two glutathione analogs, *S*-methylglutathione and *S*-propylglutathione and two other γ-glu-

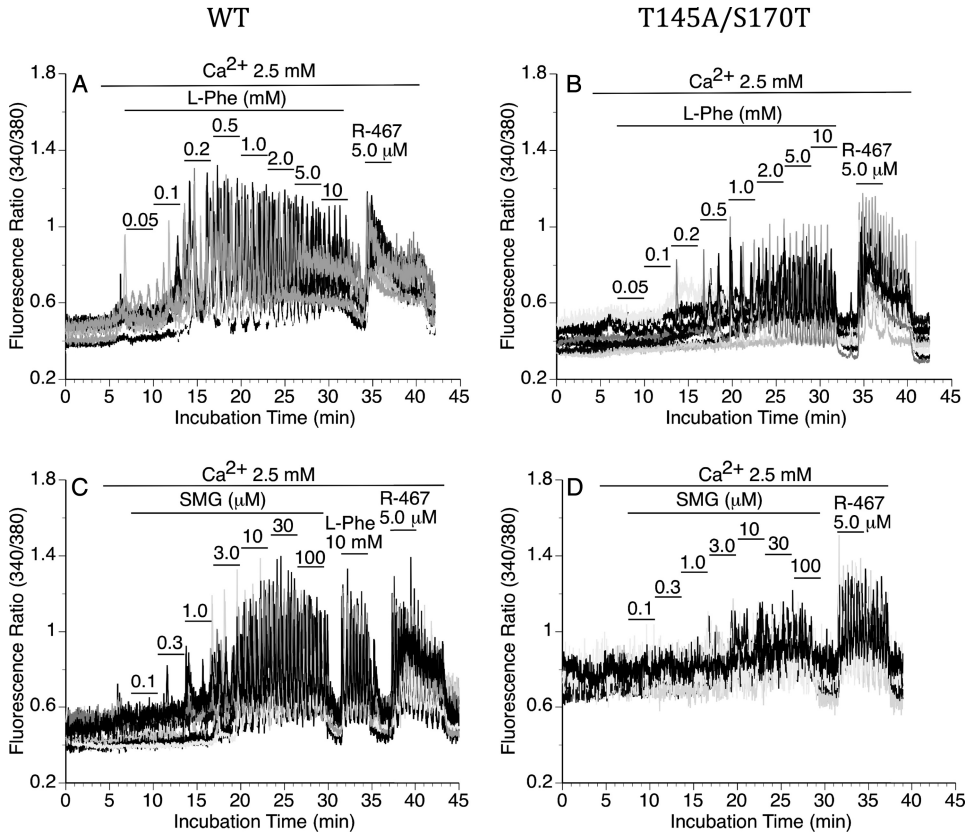

FIGURE 7. **Sensitivity of double mutant (T145A/S170T) CaR to concentration-dependent modulation of $Ca^{2+}{}_o$-dependent $Ca^{2+}{}_i$ mobilization by L-Phe or *S*-methylglutathione.** HEK-293 cells that stably expressed either the wild-type CaR (*panels A* and *C*) or the double mutant T145A/S170T CaR (*panels B* and *D*) were loaded with fura-2AM and transferred to the light path of a microfluorescence apparatus as described under "Experimental Procedures." After pre-equilibration at 0.5 mM $Ca^{2+}{}_o$, the $Ca^{2+}{}_o$ concentration was elevated to 2.5 mM. The cells were then exposed to stepwise changes in the concentrations of L-Phe (0.05–10 mM; *A* and *B*) or SMG (0.1–100 μM; *C* and *D*) followed by positive controls including the type-II calcimimetic NPS R-467 (*panels A*, *B*, and *D*) or L-Phe and R-467 (*panel C*).

tamyl peptides, γ-Glu-Ala (Figs. 1 and 3; Tables 1 and 2) and γ-Glu-Cys (Table 1) are positive modulators of CaR function, and establish that these compounds are effective inhibitors of PTH secretion (Fig. 4; supplemental Fig. S1). Of the compounds tested, *S*-methylglutathione exhibited the highest potency activation of $Ca^{2+}{}_i$ mobilization in human parathyroid cells ($EC_{50} \approx 0.5$ μM at $Ca^{2+}{}_o = 1.5$ mM; Fig. 3*C*) and inhibition of PTH secretion ($IC_{50} \approx 5$ μM at $Ca^{2+}{}_o = 1.0$ mM; Fig. 4*C*). The results suggest that extracellular glutathione or one of its metabolites, including perhaps, the natural metabolite *S*-methylglutathione, may modulate CaR function in a physiologically relevant manner. The results also identify lead compounds from which new groups of high potency activators or inhibitors of the CaR may emerge. The finding that maximally effective concentrations of *S*-methylglutathione and NPS R-467 exhibit positive interactions on the receptor $Ca^{2+}{}_i$ mobilizing response (Fig. 2) supports this notion and indicates that combination therapy with cinacalcet and an agent that targets the glutathione binding site in the receptor's VFT domain may be an option in clinical situations in which cinacalcet alone is ineffective.

The CaR negatively regulates cAMP levels in parathyroid cells (review: Ref. 2) and CaR-expressing HEK-293 cells in which both $G_{i/o}$ and $Ca^{2+}{}_i$-dependent mechanisms have been identified (27). In the current study, we transiently expressed a FRET-based cAMP sensor, CFPnd-EPac1-cpVenus (33) in CaR-expressing HEK-293 cells to permit detection of changes in intracellular cAMP levels in real-time. As expected, resting levels of cAMP were promptly elevated upon exposure of CaR-expressing HEK-293 cells to forskolin and, in its continuing presence, were suppressed in a concentration-dependent manner upon exposure to elevated $Ca^{2+}{}_o$ (Fig. 9*A*). In addition, the positive allosteric modulators L-Phe and *S*-methylglutathione (Fig. 9*B*), as well as NPS R-467 (not shown) all reversibly suppressed cAMP levels in the presence of 1.5 mM $Ca^{2+}{}_o$. Thus, the positive allosteric modulators of the CaR, L-Phe, and *S*-methylglutathione both effectively suppressed cAMP levels. It is not yet known whether these effects arose primarily from activation of $G_{i/o}$ or $Ca^{2+}{}_i$ mobilization. Previous studies have suggested that L-Phe primarily promotes a CaR-dependent pathway linked to $Ca^{2+}{}_i$ mobilization (34, 35) although it also has a small positive modulatory effect on ERK1/2 phosphorylation (36).

Chimeric receptor analysis previously demonstrated that the binding sites for L-amino acids (14) and glutathione (21) both localize to the receptor VFT domain. To investigate whether these two classes of positive allosteric modulator might bind at a common or overlapping site and/or act via a common mechanism, as suggested by molecular modeling analysis (18), we took advantage of a previously identified double mutant of the CaR VFT domain (T145A/S170T) that selectively disables L-amino acid sensing with

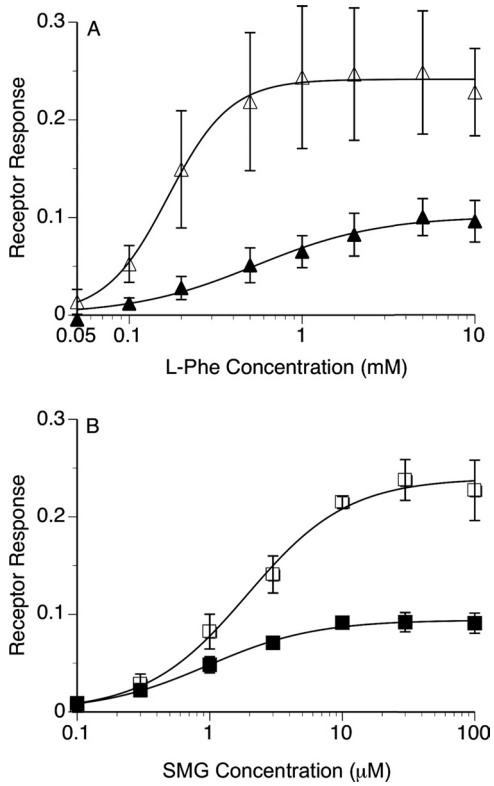

FIGURE 8. **Behavior of CaR double mutant (T145A/S170T) in concentration-response analyses for L-Phe or *S*-methylglutathione-induced $Ca^{2+}{}_i$ mobilization in the presence of a submaximal $Ca^{2+}{}_o$ concentration.** HEK-293 cells that stably expressed either the wild-type CaR (*open symbols*) or the double mutant T145A/S170T CaR (*filled symbols*) were loaded with fura-2AM and transferred to the light path of a microfluorescence apparatus as described under "Experimental Procedures." After a short pre-equilibration at 0.5 mM, the $Ca^{2+}{}_o$ concentration was elevated to 2.5 mM. *Panel A* shows the L-Phe concentration-response relations for the wild-type CaR (*open triangles*; $n = 3$) and CaR double mutant T145A/S170T (*filled triangles*; $n = 5$). The difference in the maximal responses was statistically significant ($p = 0.03$). *Panel B* shows the SMG concentration-response relations for the wild-type CaR (*open squares*; $n = 3$) and CaR double mutant T145A/S170T (*filled squares*; $n = 5$). The difference in the maximal responses was statistically significant ($p = 0.02$).

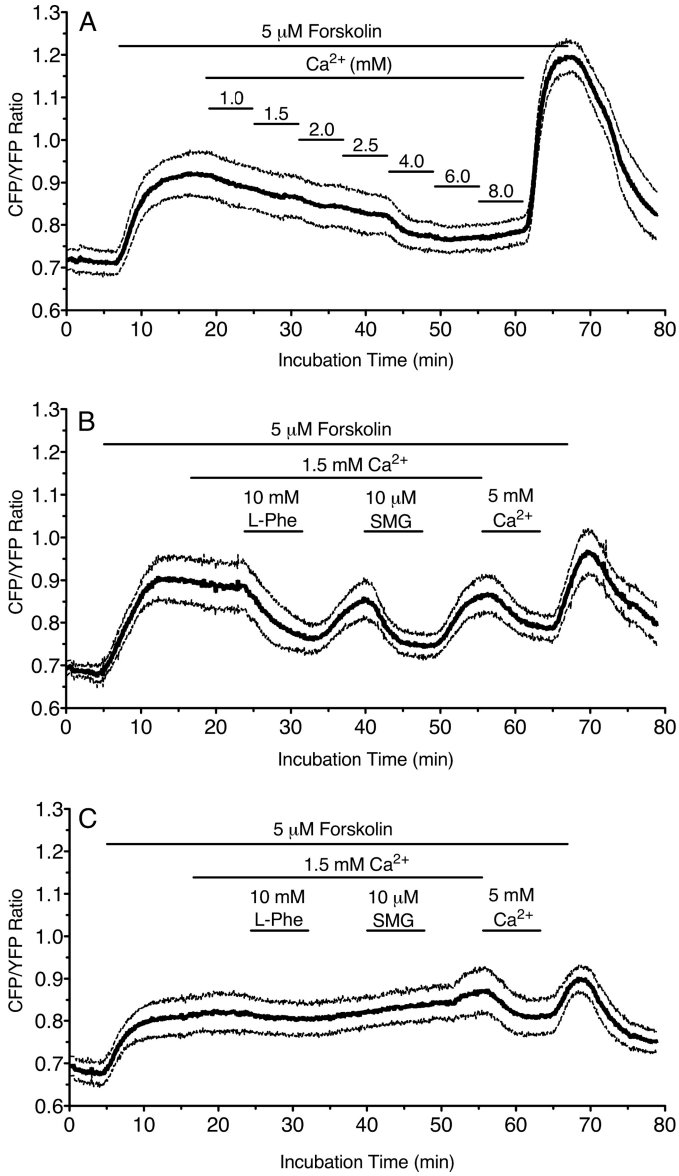

FIGURE 9. **Impacts of extracellular Ca^{2+}, L-Phe and *S*-methylglutathione on cAMP levels in HEK-CaR cells and HEK-CaR-T145A/S170T cells.** HEK-CaR cells (*panels A* and *B*) or HEK-CaR-T145A/S170T cells (*panel C*) were transiently transfected with the cAMP FRET reporter CFPnd-EPac1-cpVenus as described under "Experimental Procedures." Intracellular cAMP levels (means ± S.E.) were elevated using the cell-permeable adenylyl cyclase activator forskolin (5 μM) and the impacts of various CaR activators including $Ca^{2+}{}_o$ (0.5–8.0 mM), 10 mM L-Phe and 10 μM SMG were then investigated. HEK-293 cells that expressed the wild-type CaR exhibited $Ca^{2+}{}_o$-concentration-dependent reversible inhibition of cAMP levels; maximum inhibition was observed at around 6 mM (*panel A*; $n = 4$). In these experiments, an overshoot was commonly observed upon return to the control physiological saline solution containing 0.5 mM $Ca^{2+}{}_o$ and 5 μM forskolin. In addition, in the presence of 1.5 mM $Ca^{2+}{}_o$, 10 mM L-Phe, or 10 μM SMG reversibly suppressed intracellular cAMP levels in HEK-CaR cells (*panel B*; $n = 4$). In HEK-CaR-T145A/S170T cells, however, the effects of L-Phe and SMG were markedly attenuated whereas the effect of elevated $Ca^{2+}{}_o$ was unchanged (*panel C*; $n = 3$).

little or no effect on $Ca^{2+}{}_o$-sensing (15). This mutant disabled the effects of L-Phe as well as *S*-methylglutathione, on $Ca^{2+}{}_i$ mobilization (Figs. 5–8) as well as suppression of forskolin-elevated cAMP levels in CaR-expressing HEK-293 cells (Fig. 9). However, in the current study, the double mutant appeared to markedly impair efficacy with little or no effect on potency in the cases of both L-Phe and S-methylglutathione (Fig. 8) suggesting that its primary impact was not on ligand binding but on the coupling of the amino acid and γ-glutamyl peptide binding sites to $Ca^{2+}{}_o$-dependent receptor activation. This suggests that the mutant receptor is unable to sustain a normal conformational response to either amino acid or peptide binding with impacts on both $Ca^{2+}{}_i$ mobilization and cAMP signaling pathways. The impact on other CaR-linked signaling pathways remains to be determined. Although these results do not allow a firm conclusion to be drawn regarding the relationship between the binding sites for L-amino acids and glutathione analogs, molecular modeling suggests that the *S*-methylglutathione binding site in the CaR (supplemental Fig. S2) is closely related to that previously described for the amino acid L-glutamate in mGlu-1 (16), mGlu-3, and mGlu-7 (17). Thus, L-amino acids and glutathione analogs, distinct from $Ca^{2+}{}_o$ and R-467, act via a common mechanism as revealed by the selective impacts of the T145A/S170T double mutant and are likely to occupy overlapping binding sites in the receptor VFT domain.

Global and conditional knock-out studies for assessing CaR function have, thus far, identified roles for the CaR in the control of parathyroid hormone secretion (3, 4), renal Ca^{2+} reabsorption (3), and the formation of cartilage and bone (4). However, the realization that an earlier global knock-out strategy, based on the in-frame deletion of exon-5, was incomplete due to residual receptor function (4), together with studies demonstrating diverse pluripotent roles in development and tissue function (review: Ref. 37) suggest that the discrete list above understates the physiological importance of the receptor due to its wide distribution and expression in compartments that differ in their ionic, pH and nutrient compositions (reviews: Refs. 1, 5, 38). The current work, demonstrating that L-amino acids as well as glutathione and its metabolites modulate the CaR by a common mechanism, raises the possibility that the CaR may be modulated primarily by L-amino acids in some compartments and by γ-glutamyl peptides in others, depending on their relative levels in the compartment concerned. For example, total L-amino acid levels normally exceed 2.5 mM in systemic plasma and are even higher in the gut lumen following the ingestion of protein-rich foods (review: Ref. 19) and recent work has demonstrated the physiological significance of the CaR in luminal amino acid-induced gastrin secretion *in vivo* (39). However, L-amino acid levels are substantially lower in the cerebrospinal fluid (around 10% of those in plasma) as a result of blood-brain barrier function (1, 40). The extracellular levels of glutathione, on the other hand, appear to be higher in the brain, where they can approximate 10–20 μM due to astrocyte-dependent synthesis and release (41, 42). These considerations raise the possibility that glutathione and/or a metabolite such as *S*-methylglutathione may play a specific role in CaR-dependent neuromodulation.

The CaR belongs to GPCR class C, members of which are typified by N-terminal Venus FlyTrap domains linked to heptahelical domains. In mammalian biology, VFT domains are also found in ionotropic, as well as metabotropic, receptors for glutamate. Interestingly, glutathione and analogs including *S*-methylglutathione and glutathione sulfonate have been previously shown to modulate NMDA and AMPA receptors (43, 44). In addition, like the CaR, the NMDA receptor binds multivalent cations including neomycin, spermine, and Mg^{2+} (45). The current work on the physiological impact of γ-glutamyl peptides on CaR function strengthens the notion that the CaR and ionotropic, as well as metabotropic, glutamate receptors share conserved VFT domain nutrient binding surfaces and may thus contribute to coordinate control of CNS function in response to a common signal including, perhaps, extracellular glutathione and/or glutathione analogs. Attention has been drawn previously to potential roles for glutathione and its metabolites in the modulation of CNS function (reviews: Refs. 46, 47).

In conclusion, we have demonstrated that γ-glutamyl peptides including glutathione and its *S*-alkyl derivatives, *S*-methylglutathione, and *S*-propylglutathione along, with the di-peptides γ-Glu-Ala and γ-Glu-Cys are potent positive allosteric modulators of the calcium-sensing receptor that stimulate $Ca^{2+}{}_i$ mobilization in CaR-expressing HEK-293 cells and normal human parathyroid cells and powerfully suppress PTH secretion. In addition, they suppress cAMP levels in forskolin-stimulated CaR-expressing HEK-293 cells transfected with the cAMP sensor CFPnd-EPac1-cpVenus. Finally, unlike responses to elevated $Ca^{2+}{}_o$ or the type-II calcimimetic NPS R-467, both γ-glutamyl peptide-induced $Ca^{2+}{}_i$ mobilization and suppression of cellular cAMP levels were markedly attenuated in the case of the CaR double mutant T145A/S170T indicating that L-amino acids and γ-Glu peptides activate the CaR via a common mechanism.

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